Sister-chromatid cohesion via MEI-S332 and kinetochore assembly are separable functions of the *Drosophila* centromere

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Attachment, or cohesion, between sister chromatids is essential for their proper segregation in mitosis and meiosis [1,2]. Sister chromatids are tightly apposed at their centromeric regions, but it is not known whether this is due to cohesion at the functional centromere or at flanking centric heterochromatin. The *Drosophila* MEI-S332 protein maintains sister-chromatid cohesion at the centromeric region [3]. By analyzing MEI-S332's localization requirements at the centromere on a set of minichromosome derivatives [4], we tested the role of heterochromatin and the relationship between cohesion and kinetochore formation in a complex centromere of a higher eukaryote. The frequency of MEI-S332 localization is decreased on minichromosomes with compromised inheritance, despite the consistent presence of two kinetochore proteins. Furthermore, MEI-S332 localization is not coincident with kinetochore outer-plate proteins, suggesting that it is located near the DNA. We conclude that MEI-S332 localization is driven by the functional centromeric chromatin, and binding of MEI-S332 is regulated independently of kinetochore formation. These results suggest that in higher eukaryotes cohesion is controlled by the functional centromere, and that, in contrast to yeast [5], the requirements for cohesion are separable from those for kinetochore assembly.

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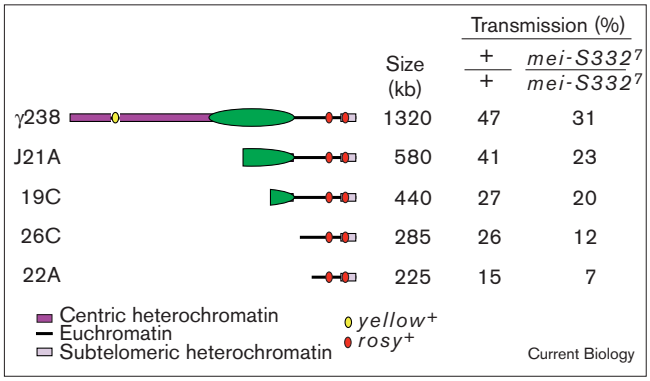
Results and discussion

Analyses of the structures and transmission behavior of minichromosome derivatives defined a functional centromere in *Drosophila* [5,6]. We tested whether MEI-S332 was necessary for transmission of the minichromosomes by measuring transmission frequencies in *mei-S332*^{7} mutants which produce a truncated protein that is not functional [3,7]. Loss of MEI-S332 function results in random segregation of endogenous chromosomes at meiosis II [8], and thus should decrease transmission of a minichromosome.

We found that the frequency of transmission of all of the minichromosome derivatives was significantly decreased in a *mei-S332*^{7} homozygous mutant background (summarized in Figure 1; for data and controls see Supplementary material). Thus, MEI-S332 function is necessary for normal minichromosome transmission.

By analyzing MEI-S332 localization on rearranged chromosomes with displaced centric heterochromatin we found that MEI-S332 is not present throughout the heterochromatin (see Supplementary material). We used the minichromosomes to map chromosomal sites of MEI-S332 localization more precisely by comparing the requirements for MEI-S332 minichromosome localization with those for kinetochore assembly. Transmission frequencies decline significantly in the derivatives in which core components of the centromere are deleted ([5], and Figure 1). Nevertheless, all of the minichromosome derivatives assemble a

Figure 1



Structure and behavior of *γ238* and deletion derivatives. Both the total size of the minichromosome (in kb) and the male transmission frequencies (%), in wild-type flies and *mei-S332* mutants are listed to the right. The mutants have significantly reduced levels of transmission of all the minichromosomes. The data and the statistical analysis can be found in the Supplementary material. The functional centromere was defined by transmission frequencies in females, which are more sensitive to compromised centromere activity [4], and this DNA is deleted in the *19C*, *26C* and *22A* derivatives [4,5]. Two minichromosome derivatives used in this study, *20A* and *10B*, are larger than *J21A* and have complete centromeres [4].

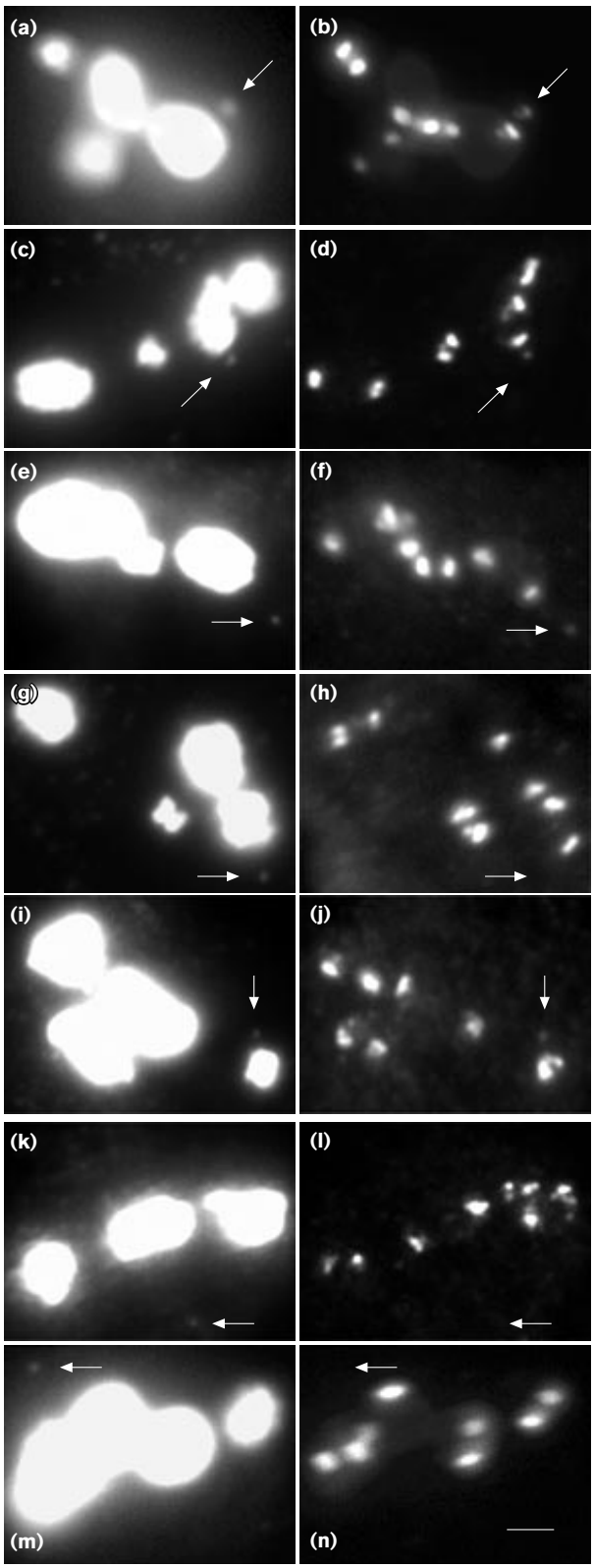


Figure 2

Localization of MEI-S332 on minichromosomes. Prometaphase I spermatocytes containing minichromosome derivatives were stained with DAPI (left column) and MEI-S332 antibodies (right column). Cells containing the derivatives **(a–b)** *γ238* and **(c–d)** *J21A* show MEI-S332 localization to the minichromosomes (arrows) in all spermatocytes examined. Cells containing the derivatives **(e–h)** *19C*, **(i–l)** *26C* and **(m,n)** *22A* show decreasing frequency of MEI-S332 localization to the minichromosomes (arrows), with 62% in (e–h), 45% in (i–l), and none in (m,n). In (e–h) and (i–l) an example is shown of a minichromosome with MEI-S332 localized and one lacking MEI-S332. For each of these minichromosomes at least 20 spermatocytes containing the minichromosome were tested for MEI-S332 localization. The DAPI images have been overexposed to visualize the minichromosomes, making the individual large chromosomes indistinguishable. The scale bar represents 1 μm.

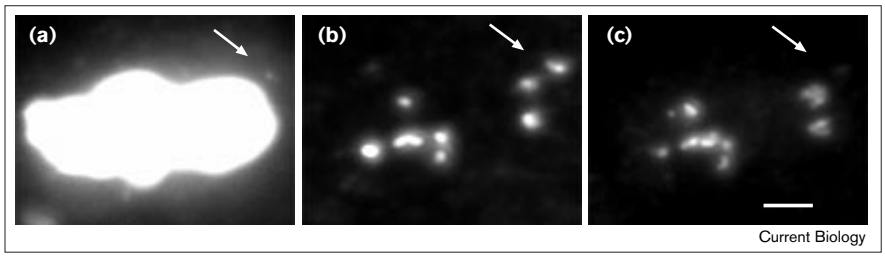
kinetochore, as evidenced by staining with antibodies against the ZW10 and dynein kinetochore proteins [9,10].

Indirect immunofluorescence analysis of spermatocytes using MEI-S332 antibodies demonstrated that *γ238*, *20A*, *10B*, and *J21A* do associate with MEI-S332 (Figure 2a–d, and data not shown). In every cell scored MEI-S332 was present on the minichromosome. These results indicate that MEI-S332 associates consistently with minichromosomes that contain fully functional centromeres, and is not present exclusively in the centric heterochromatin that flanks the centromere.

When we assayed the association of MEI-S332 with less functional minichromosome deletion derivatives we found some deletions affected MEI-S332 localization, despite having no effect on the localization of kinetochore proteins. The frequency of MEI-S332 staining was correlated with the reduced transmission frequencies of more unstable minichromosomes. We detected MEI-S332 on 62% of the *19C* minichromosomes and on 45% of the *26C* minichromosomes (Figure 2e–l). We were unable to detect MEI-S332 on the smallest derivative, *22A* (Figure 2m,n).

As a positive control, we double labeled *22A* spermatocyte preparations with MEI-S332 and antibodies to a known kinetochore protein, ZW10 [9]. MEI-S332 was undetectable on *22A*, while ZW10 staining was normal (Figure 3). Thus, deletion of the core centromere sequences abolishes the ability to detect MEI-S332 localization by immunofluorescence, but does not diminish the localization of a kinetochore protein. In addition to these differences in the frequency of minichromosomes stained with anti-MEI-S332 or anti-ZW10 antibodies, there is also a distinction in the intensity of staining. When we quantified the intensity of the fluorescence signal at the MEI-S332 focus on the minichromosome relative to the bivalents in the spermatocytes, we found that the intensity within the spot was between half and equal to that seen on the bivalents (see Supplementary material). The size of the spot was considerably smaller than on the normal chromosomes. In contrast, ZW10 gave comparable staining on all the minichromosomes relative to the other chromosomes [9].

Figure 3



Localization of ZW10 but not MEI-S332 on *22A*. A prometaphase I spermatocyte containing minichromosome *22A* (arrow) stained with **(a)** DAPI, **(b)** ZW10 antibodies and **(c)** MEI-S332 antibodies. ZW10, but not MEI-S332, localizes to *22A*. The scale bar represents 1 μm.

MEI-S332 localization on the minichromosome derivatives correlated with the presence of a functional centromere. Because the transmission frequency to minichromosome size ratio is not strictly linear [5], the decrease in centromere function and MEI-S332 localization cannot be strictly due to a decreasing size effect. For example, the *γ238* derivative is much larger than the *10B* derivative, by ~600 kilobases (kb), yet the transmission frequencies of these minichromosomes are the same [5] and the levels of MEI-S332 also appear to be the same (data not shown). Additionally, there is only a slight difference in size (~60 kb) between the severely compromised derivatives, *26C* and *22A*, yet we are able to detect MEI-S332 on *26C* nearly half the time, but MEI-S332 was not detected on *22A*. Transmission of *22A* is reduced in *mei-S332* mutants, so there may be low levels of MEI-S332 bound that facilitate transmission yet are not detectable by immunofluorescence. It makes sense that MEI-S332 localization is very tightly linked to a functional centromere, because this is the site at which sister-chromatid cohesion would be most critical to ensure proper segregation.

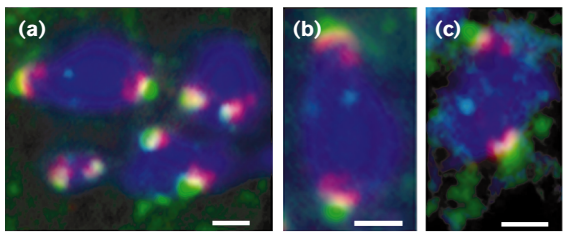
Though MEI-S332 is functionally linked to the centromere, it is unlikely that specific primary DNA sequences dictate its localization or function, and instead a specific chromatin structure may be crucial. This has been suggested for centromere function in general [11,12]. MEI-S332 localizes on the 26C minichromosome which has only X euchromatic and subtelomeric DNA from regions that never bind MEI-S332 in their normal context.

ZW10, dynein and MEI-S332 are localized to the centromeric region in double-labeled spermatocytes; the signals, however, are not overlapping. MEI-S332's location appears distinct from the outer kinetochore plate, the likely location of the ZW10 and dynein proteins [10]. Dynein is a microtubule motor protein localized to the fibrous corona of mammalian kinetochores [13–15] and to *Drosophila* prometaphase I spermatocyte kinetochores [10]. Double labeling of spermatocytes with MEI-S332 and ZW10 or MEI-S332 and dynein revealed that the ZW10/dynein localization was mostly found on the outer edge of the bivalent, presumably oriented in the direction of microtubules emanating from the poles, whereas MEI-S332 was on the inner bivalent mass on the chromatin side (Figure 4).

Studies of mammalian cells show that segments of the kinetochore can be resolved at a comparable level of immunofluorescence and can reveal the position of various kinetochore proteins with the same precision predicted from immunoelectron microscopic analysis [16,17]. The adjacent but non-overlapping signals of MEI-S332 and ZW10 or dynein indicate that MEI-S332 is not a component of the outer region of the kinetochore and instead may associate either with the inner plate of the kinetochore, the structure that is closely associated with the DNA [16,17], or the centromere chromatin. Electron microscopic immunolocalization analyses are required to confirm this finding at the ultrastructural level.

Does kinetochore assembly direct sister-chromatid cohesion at the centromere? It is difficult to imagine that the

Figure 4



Localization of MEI-S332 compared to ZW10 and dynein. **(a)** A prometaphase I spermatocyte stained with ZW10 antibodies (green), MEI-S332 antibodies (red), and DAPI (blue) shows that MEI S332 and ZW10 localize to the centromeric regions of the bivalents, but their staining does not overlap. **(b)** ZW10 (green) and MEI-S332 (red) localization on a single bivalent, with chromatin in blue. MEI-S332 is closer to the chromatin than ZW10. **(c)** MEI-S332 (red) is also closer to the chromatin (blue) than dynein (green). For all the images, the registration between the channels was adjusted using Cy3- and Cy2-conjugated beads. On all the chromosomes the ZW10 and dynein signals are external to the chromosome mass on both sides of the bivalents, whereas MEI-S332 is adjacent to the chromatin. The scale bar represents 4 μm.

kinetochore is involved in the establishment of cohesion during S phase, but maintenance of cohesion in mitosis and meiosis II could be linked to kinetochore assembly. All the compromised minichromosomes bind the ZW10 and dynein kinetochore proteins at frequencies and intensities analagous to endogenous centromeres, and thus appear to assemble kinetochores [9,10]. Furthermore, these minichromosomes are often seen leading to the poles, which indicates that they bind microtubules [9]. This implies that a functional kinetochore has formed, yet they have a reduced ability to localize MEI-S332. Thus, in contrast to the simple centromere in budding yeast [5], in *Drosophila* cohesion and kinetochore function appear to be distinct. Our observations suggest that the instability of minichromosomes with compromised centromeres is due at least partly to aberrant cohesion. Possibly MEI-S332 is a *Drosophila* version of a cohesin or is involved in protecting the cohesin complex at the centromere. Identification of the *Drosophila* cohesin subunits and analyzing their localization on the minichromosome derivatives may help distinguish between these possibilities.

Materials and methods

All minichromosome stocks were described and maintained as in [5]. The transmission of all minichromosome derivatives tested here was determined as previously described in [5]. Two stocks of the *mei-S332*^{7} allele were generated: *y*; *pr cn mei-S332*^{7} *bw sp*/*SM1*; *ry; Dp* and *y*/*y*$^{+}$*Y*; *cn mei-S332*^{7} *px sp*/*SM1*; *ry.* These stocks were crossed, and the *Dp*-containing male progeny *y*; *pr cn mei-S332*^{7} *bw sp*/*cn mei-S332*^{7} *px sp*; *ry; Dp* were selected to assay mutant effects on the *Dp*s. For wild-type controls *y*; *pr cn mei-S332*^{7} *bw sp*/*SM1*; *ry; Dp*, *y*; *cn mei-S332*^{7} *px sp*/*SM1*; *ry; Dp* or *y; ry; Dp* males were used.

Testes from animals carrying the minichromosomes were squashed and stained with guinea pig MEI-S332 antibodies as in [3,18]. The secondary antibodies were either Cy3- or Cy2-conjugated anti-guinea pig, Cy3-conjugated anti-rabbit and Cy3-conjugated anti-mouse (Jackson ImmunoResearch Laboratories). The rabbit ZW10 and mouse antibodies were kindly provided by Michael Goldberg and Thomas Hays, respectively.

Microscopic examination of the minichromosome samples was done on a Nikon Eclipse E800 epifluorescent microscope equipped with a Nikon 100× oil objective using a Photometrics CE200A cooled CCD video camera. For the ZW10 and dynein colocalization studies with MEI-S332 a Nikon TE300 inverted epifluorescent microscope with a Hamamatsu CCD was used. The images were processed with Metamorph imaging software and then with Adobe Photoshop 5.0. We eliminated registration differences between the Cy3 and Cy2 channels by imaging beads labeled with both dyes (Molecular Probes). The two channels were imaged separately and superimposed using the Metamorph software. This required a shift of two pixels (0.134 μm) on the *x* axis and one pixel (0.067 μm) on the *y* axis. The spermatocyte samples were then imaged with identical filter sets, lens, and binning number and the two channels adjusted as for the bead standards.

Supplementary material

Supplementary material including analysis of MEI-S332 localization on rearranged chromosomes and the transmission data and statistics is available at http://current-biology.com/supmat/supmatin.htm.

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